PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/31, C07K 14/335, C12N 15/62, C07K 16/12, A61K 39/07, G01N 33/50, A23L 1/00

A1

(11) International Publication Number: WO 97/14802

(43) International Publication Date: 24 April 1997 (24.04.97)

(21) International Application Number: PCT/NL96/00409

(22) International Filing Date: 21 October 1996 (21.10.96)

(30) Priority Data:
PCT/NL95/00367 20 October 1995 (20.10.95) WO
(34) Countries for which the regional or international application was filed: NL et al.

(71) Applicant *(for all designated States except US)*: NEDERLANDSE ORGANISATIE VOOR TOEGEPAST-NATUURWETENSCHAPPELIJK ONDERZOEK TNO [NL/NL]; Schoemakerstraat 97, NL-2628 VK Delft (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: LEER, Robert, Jan [NL/NL]; Tulpentuin 36, NL-2272 XK Voorburg (NL). POUWELS, Pieter, Hendrik [NL/NL]; Delftweg 14, NL-2289 AJ Rijswijk (NL). CONWAY, Patricia, Lynne [AU/AU]; 22 Goorawahl Avenue, Le Perouse, NSW 2036 (AU).

(74) Agent: DE BRUIJN, Leendert, C.; Nederlandsch Octrooibureau, Scheveningseweg 82, P.O. Box 29720, NL-2502 LS The Hague (NL).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: NOVEL ADHERENCE FACTORS OF NON PATHOGENIC MICROORGANISMS AND APPLICATIONS THEREOF FOR SCREENING MICROORGANISMS FOR SPECIFIC PROBIOTIC PROPERTIES; NOVEL PHARMACEUTICAL COMPOSITIONS AND FOOD ADDITIVES COMPRISING SUCH MICROORGANISMS AND ADHERENCE FACTORS

(57) Abstract

A protein obtainable from a non pathogenic microorganism, said protein having mucosa binding promoting activity and a molecular weight of 20-40 kD is disclosed. Application of such a protein or a peptide derived therefrom in a method of screening non pathogenic microorganisms for a microorganism capable of specifically binding mucosa, said method comprising detection in a manner known per se of the presence of a particular protein on or in a microorganism or in a culture of microorganisms, said particular protein being the already defined protein. Kits suitable for such a screening method are also disclosed. Use of a component selected from the group of components comprising a protein or peptide as defined; an expression vector comprising nucleic acid encoding such protein or peptide; a recombinant microorganism or a part of said microorganism expressing such protein or peptide, said part expressing mucosa binding promoting activity; a non pathogenic microorganism capable of expressing such protein or peptide or a part of said microorganism, said part expressing mucosa binding promoting activity as pharmaceutically active component in a pharmaceutical composition for prophylaxis and/or treatment of disease or illness associated with a mucosa colonising pathogenic microorganism. Use of such components as food additive and compositions comprising such components are described.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

Novel adherence factors of non pathogenic microorganisms and applications thereof for screening microorganisms for specific probiotic properties; novel pharmaceutical compositions and food additives comprising such microorganisms and adherence factors

SUMMARY OF THE INVENTION

This invention relates to the screening of bacteria, in particular non pathogenic bacteria for those bacteria that can adhere to specific sites of the mucosa called receptors. More specifically the invention is directed at screening of non pathogenic Gram positive bacteria in particular lactic acid bacterial (LAB) species, more in particular bacteria of the genera *Lactobacillus* and *Bifidobacterium*. A preference is expressed for screening bacteria indigenous to farm animals, pets and humans.

The invention comprises a method of screening for a particular group of adherence factors of the non pathogenic bacteria not previously recognised. In particular the adherence factors e.g. of Lactobacilli are of interest. This novel group of adherence factors of non pathogenic bacteria comprises proteins that are structurally related to virulence factors of certain classes of pathogenic microorganisms.

The invention also relates to the application of bacteria obtainable via the screening method of the invention, in particular to Lactobacilli producing said adherence factors, application of the adherence factors as such, application of parts of the bacteria and application of parts of an adherence factor from the novel group for various pharmaceutical applications. Such application may comprise the treatment or prophylaxis of infections of the gastro-intestinal tract, the respiratory tract, urogenital tract, the oral cavity or any other part of the body in particular any internal part of the body that can be colonised by pathogenic microorganisms.

Another suitable example of application comprises the targeting of specific compounds to cells of the mucosa, for example with the aim to evoke a specific mucosal immune response against said compound, or to modulate the immune response.

Novel microorganisms obtainable e.g. through recombinant DNA technology expressing or overexpressing any of the novel adherence factors or effective parts thereof are also included within the invention.

The nucleic acid sequences encoding the adherence factors and fragments of said sequences encoding mucosa binding expression products are also part of the invention as are the recombinant products resulting from expression of said nucleic acid sequences.

Novel pharmaceutical compositions comprising the nucleic acid or expression products thereof or microorganisms expressing or overexpressing an adherence factor of the novel type also fall within the scope of the invention.

BACKGROUND TO THE INVENTION

Pathogenic viruses and bacteria can adhere to specific sites of the mucosa, called receptors and invade the underlying cells *via* these receptors, resulting in illness or even the death of the host organism. For public and animal health care it is essential that effective and cheap means are available to prevent and/or cure infectious diseases in humans and animals.

The mucosa form the porte d'entrée of numerous pathogenic bacteria, for example of Gram negative bacteria of the genera *Escherichia*, *Campylobacter*, *Haemophilus*, *Shigella*, *Vibrio*, *Pasteurella*, *Yersinia*, *Salmonella*, Gram positive bacteria like *Mycobacterium*, *Listeria*, *Clostridium*, *Staphylococcus* and viruses like rotavirus, poliovirus, measles and many other microorganisms well known to a person skilled in the art of microbial infections.

Bacteria of the genus *Campylobacter* for example can cause severe enteritis in humans and animals after oral ingestion. *C.jejuni* is a major cause of diarrhoea in humans and occasionally in animals. Beside diarrhoea, *C.jejuni* can occasionally also cause appendicitis, meningitis, abortion and urinary tract infection in humans. In developed countries, persons of all ages are affected and *Campylobacter* infections are as common as infections caused by *Salmonella*, *Shigella* or *Vibrio cholerae*.

Mycobacteria such as *Mycobacterium tuberculosis* and *Mycobacterium leprae* also cause serious diseases such as tuberculosis and lepra respectively. These bacteria cause the death of many individuals in particular in the less well developed countries. These microorganisms invade the body via the mucosa of the respiratory tract.

Pathogenic microorganisms can adhere to parts of the body e.g. the gastro-intestinal tract, thereby initiating a disease. The studies of

the adhesion of pathogenic microorganisms to parts of the body of a host organism have resulted in a wealth of data. From these studies it has become clear that adhesion of pathogenic bacteria can be mediated by proteins. Detailed information is available about proteins from pathogenic bacteria that bind to components of the extra cellular matrix, e.g. collagens, fibronectin or proteoglycans. Particular examples are the mycobacterial fibronectin-binding proteins, the fibronectin- and collagen-binding proteins of Streptococci and Staphylococci, specific enterobacterial fimbrial types, and surface proteins of Yersinias and the A-protein of *Aeromonas* (for a review, see Westerlund and Korhonen, Mol. Microbiol. 9:687-694 1993).

Information about the adhesion of Gram-positive, non pathogenic bacteria to surfaces of a host organism is more limited, in particular information regarding specific binding of mucosal receptors by non pathogenic microorganisms is scarce.

It is common knowledge that the normal human gastro-intestinal tract is colonized by a variety of non pathogenic microorganisms including bacteria of the genera *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Bifidobacterium*, *Clostridium*, *Bacteroides*, and others. These microorganisms form part of the indigenous microflora of the human being. As such considerable interest has been directed to elucidating the mechanisms of adherence and the role of adhesion in gastro-intestinal colonisation. However the mechanisms of adhesion of LAB, a well examined group of non pathogenic bacteria present in gut microflora of humans and animals are in general more complex than those of the gastro-intestinal pathogens (Hasty et al. Infect. Immun. 60:2147-2152 1992).

Adhesion of non-pathogenic bacteria may be specific or aspecific. Hydrophobic and electrostatic adhesion mechanisms are involved in non-specific adhesion. Specific adhesion is characterized by a so-called "lock-and key mechanism", in which the adherence factor binds to a specific receptor. Specific adhesion is usually associated with the adhesion of microorganisms to receptors on living tissues. Adherence factors or adhesins are, in general, surface bound molecules. The adhesin can be firmly attached to the surface of the bacterium or loosely bound. The receptor is a component or structure on the surface of the cell where the bacterium will bind by an active site of the adhesin (Rutter et al, 1984 Mechanisms of adhesion *in* "Microbial adhesion and aggregation" Marshall, K.C. ed. pp 5-19, Springer-Verlag, Berlin).

Lactic acid bacteria, particularly *Lactobacillus* and

Enterococcus, are examples of non pathogenic Gram-positive bacteria that play a key role in the establishment and maintenance of the microflora of the gastro-intestinal tract of man and animals. *Lactobacillus* species have been isolated from various regions of the human gastro-intestinal tract (Molin et al, J. Appl. Bacteriol. 74, 314-323 1993).

The determinants supposedly responsible for the adhesion of some strains have been studied, and certain structures are reported to be involved in the mechanism of adhesion. However, because of the complexity of the intestinal ecosystem, little is known about why and how certain bacterial strains adhere to and colonize specific regions of the gastro-intestinal tract.

There is indeed great confusion in the literature about the mechanisms of adhesion of Lactobacilli to the gastro-intestinal mucosa. Fuller described the adhesion of *Lactobacillus* to chicken crop epithelium and concluded that the adhesion was mediated by polysaccharides (Fuller, J. Gen Microbiol. 87:245-250 1975). However, Conway and Adams (J. Gen. Microbiol. 135:1167-1173 1989), who found no evidence for a role of polysaccharides in the adhesion of lactobacilli, suggested that other components may be involved. Other researchers have indeed shown that lipotheichoic acids (LTA) are very important in the adhesion of *Lactobacillus* and *Streptococcus* and proposed that LTA is responsible for the association of Streptococci with fibronectin and dental plaque (Hogg and Manning, J. Appl. Bacteriol. 65:483-489; Vickerman and Jones, Infect. Immun. 60:400104008 1992). Suegara et al (Infect. Immun. 12:173-179) have described that proteinaceous material mediates adhesion of lactobacilli to the rat stomach epithelium.

In Current Microbiology. Vol. 28 (1994) p. 231-236 Aleljung P. et al. describe purification of 2 collagen binding proteins of *L. reuteri* NC1B 11951 which bind to collagen type I. One of 31 kD with a sequence XSNKPIIVGSK*XV. One of 29 kD with a sequence ASS*AVNSELV. The closest homology appeared to be with a trigger factor of *E. coli*. TIG position 27-33 with a relative score of 79 %. It is also stated the CnBP of *L. reuteri* do not seem to be S Protein, a protein type which has been illustrated to be involved in adhesion to chicken alimentary tract. They state "now non-pathogenic indigenous gut microflora are illustrated as binding extracellular matrix binding protein". They do not however illustrate in vitro or in vivo binding to mucosa or mucin. They merely illustrate binding to a component as such which is known to be present in mucosa. No illustration of binding to such component in the form in which

it is present in mucosa is provided. It is not clear whether such binding to collagen when present in mucosa would occur due to the fact that it is unclear where the binding site is and whether such site is available or present for binding in collagen when present in mucin or mucosa. No illustration of non-pathogenic microorganism adhesion to ECM or mucosa is provided. The article is largely speculative in nature.

Recently, Toba et al have shown that adhesion of *Lactobacillus crispatus* to the extracellular matrix is mediated by the S-layer protein (Toba et al. Appl. Environm. Microbiol. 61: 1995).

In EP 0 210 579 (with a priority date of November 1984) a preparation is described containing a protein of a Mw of 14 kD claimed to be the responsible compound for the enhancement of bacterial adhesion to squameous epithelium in mice and pigs. The preparation containing the 14 kD protein was obtained by cultivating *Lactobacillus fermentum* in a medium rich in sugars and amino acids. From EP 0 210 579 it is not clear whether the adhesion promoting factor is specific for non-pathogenic bacteria or also may enhance the adhesion of pathogens that normally do not adhere. It is also not clear from EP 0 210 579 whether or not the adhesion promoting factor enhances adhesion to specific sites (receptors) or to a-specific sites. Moreover, EP 0 210 579 does not make clear what the origin of the 14 kD protein is. It remains uncertain whether the 14 kD protein is synthesized by *L. fermentum* as such or is generated from medium components by an activity of *L. fermentum*. Thus both identity and applicability of the 14 kD protein remain obscure in the publication.

A number of later publications also suggest different proteinaceous components being involved, however offer no conclusive data.

WO 90/09398 of Conway and Kjelleberg for example describes a fraction derived from *L. crispatis* 104 of over 30 kD exhibiting anti-pathogenic activity. The fraction of over 30 kD maintains it's activity after treatment with pronase or trypsin. It is obtained by growth of *L. crispatis* in complex medium. The application also mentions that the corresponding fraction of 8000-30.000 did not exhibit anti-pathogenic activity.

The application is silent on the exact nature of the responsible component or components. It suggests also inhibiting the adhesion of pathogens to gastrointestinal epithelium of humans and animals. They also indicate in this respect that adhesion of an *E. coli* K88 strain to pig intestinal mucosa was inhibited by the high molecular

weight metabolites of Lactobacilli isolated from the pig but not of lactobacilli from the mouse digestive tract. Subsequent studies indicated that this was not the growth inhibiting compound in casu and the mechanism of inhibition of adhesion was to be investigated. Lactobacillus metabolites could perhaps inhibit pathogen colonisation of the mucosal surface which is a prerequisite for pathogenicity for many strains. Consequently factors in addition to growth inhibition activities should also be considered. No illustration is given of mucosal binding inhibition. What is illustrated is that *Lactobacillus fermentum* KLD inhibited growth of *E. coli* strains, *Campilobacter jejeuni*, *Salmonella sofia* and *Streptococcus faecium* in vitro. The supernatant derivable upon growth of the L. fermentum with glucose e.g. BHI medium followed by dialysis and fractionation over ultrafilters with a cut-off of MW of 10.000 and 30.000 is described as being able to elicit such effect.

The most recent publication of the aforemetioned nature being that of Blomberg L.; Henriksson A.; Conway P. (Appl. Env. Microbiol. feb. 91, p499-502) in which a protein-mediated adhesion mechanism of a *Lactobacillus fermentum* strain to mouse squamous epithelium, said protein being present in a retentate fraction of culture fluid with a MW higher than 250.000 is postulated. The publication is silent on the nature of the protein and explicitly states it had not been isolated and that the efficacy had to be verified by further experiments.

In conclusion: Although the role of proteins and the nature thereof in the adhesion of bacterial pathogens is undisputed and well documented, the role of proteins in adhesion of non-pathogenic bacteria is still at the least controversial and unclear.

Although many diseases can be treated with antibiotics or drugs, there is a general tendency to limit the use of such compounds, as more and more pathogenic organisms become resistent to antibiotics and drugs. A very promising alternative to drugs for treatment of intestinal diseases is the use of non-pathogenic bacteria with probiotic properties.

Probiotics are defined as "mono or mixed cultures of living organisms which, applied as dried cells or as a fermented product to humans or animals, beneficially affect the host by improving the properties of the indigenous microflora."

Some strains of *Lactobacillus* and *Bifidobacterium* strains, reportedly, have probiotic properties. The beneficial effects have been attributed to the lowering of the pH, a condition which reduces the proliferation of Gram-negative pathogens like *Escherichia coli*. In

addition, many species of lactic acid bacteria produce oligopeptides with antimicrobial properties, called bacteriocines. These compounds are bacteriostatic or bacteriocidal for Gram-positive bacterial pathogens, like *Clostridium*, *Listeria* etc.

Some Lactobacilli have been suggested as inhibiting adhesion of pathogens in animals and in *in vitro* models. These inhibitory effects are usually explained by non-specific steric hindrance of the receptors for pathogens. In contrast, each pathogen has a specific intestinal receptor (Falkow et al. Ann. Rev. Cell. Biol. 8: 333-363 1992).

Lactobacilli or preparations made with Lactobacilli are thus widely used to treat intestinal and urinary tract disorders (see e.g. WO 9 516 461; RU 2 000 116; WO 9 418 997; EP 0 577 903; GB 2 261 372; WO 9 301 823; WO 0 921 475; US 7 822 505; CA 1 298 556; EP 0 199 535; EP 0 210 579). The beneficial effects of such preparations have been attributed to various factors, but the properties and mode of action of such health stimulating compounds have either not been disclosed or are at most mere postulations. Answers regarding the mechanism of probiotics are crucial in order to find novel enhanced probiotics and optimise their use.

Considering the economic importance for food industries to use starter strains which show a scientifically proven probiotic effect, and the equally large interest of pharmaceutical companies to use GRAS (Generally Recognised As Safe) organisms as carriers for the development of mucosal vaccines, considerable effort is spent in screening bacteria, in particular GRAS organisms, more in particular Lactobacilli, for probiotic and/or immune modulating properties. A major disadvantage of the present screening programmes is that they are laborious, time-consuming and thus very costly. No easy and reliable testsystem is available to screen for probiotic or immune modulating properties of bacterial strains.

OBJECT OF THE INVENTION

The objective of the invention is to overcome the above mentioned difficulties. It is now proven unequivocally that a 29 kD proteinaceous compound is responsible for specific adhesion of *L. fermentum* to receptor sites in the mucus of pigs and mice, and thus methods to screen for other microorganisms that synthesize proteins with a similar structure and function are now provided. By demonstrating that the adherence promoting entity of *L. fermentum* is a protein of 29 kD which is structurally related to adherence factors of certain pathogenic

bacteria and by demonstrating the nucleotide sequence of the gene encoding the adherence factor, the present invention provides methods for the rapid screening of microorganisms that contain a gene coding for an adhesin of the novel type and methods for screening of microorganisms that produce such an adherence factor using standard protein and nucleic acid technologies.

By demonstrating for the first time a structural relationship between virulence factors of pathogenic bacteria and adherence factors of non-pathogenic bacteria, *i.e.* Lactobacilli, the present invention provides methods to selectively and specifically interfere with the adhesion of pathogens to receptors on the mucosa of the gastro-intestinal tract, of the urogenital tract, of the oral cavity, of the respiratory tract and of the nasal cavity and to screen microorganisms for the capacity to interfere with adhesion of the aforementioned type of pathogens.

The many applications now possible will be explained in more detail below.

i) A more rapid and directed screening of bacteria for bacteria with probiotic properties and/or immunomodulating properties is now possible. The present invention allows rapidly screening bacteria for the capacity to interfere with the adherence of pathogens to mucosal receptors. In particular, the present invention provides a method to screen microorganisms for the presence of an adherence factor that enhances the specific adhesion of non-pathogenic Gram positive bacteria, more in particular the adhesion of lactobacilli, to bacterial receptor(s) of the mucosa of the gastro-intestinal tract, the urogenital tract, the respiratory tract and the oral/nasal cavity of humans and animals. Preferably the microorganisms to be screened will be microorganisms that are non pathogenic in humans and animals. Such microorganisms will preferably be indigenous to humans and/or animals, thus already being able to withstand the environment in which they are to be applied and also obviously not being toxic to the particular species from which they are derived. Examples of suitable non pathogenic microorganisms include bacteria of the genera *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Bifidobacterium*, *Clostridium* and *Bacteroides*.

The screening can occur at protein and/or nucleic acid level using standard technologies known per se for protein detection or nucleic acid detection such as nucleic acid amplification and hybridisation techniques and protein or peptide assays using polypeptide or protein probes and/or

antibodies specific for the adherence factor or factors to be detected. The present invention thus also provides a method to screen microorganisms for the presence of nucleic acid that encodes proteins with the desired adherence properties. Protein and nucleic acid assays are readily carried out by persons skilled in the art once the relevant amino acid and nucleic acid sequences have been determined as in the instant case. Such information enables probes and primers to be constructed when the nucleic acid sequence and/or the relevant amino acid sequence of the protein has been determined and isolated or synthesized as in the instant case. The isolation of the pure protein and/or expression of the pure protein enables production of antibodies in a manner known per se.

According to the invention, bacteria can be screened for the presence of a protein falling within the definition of the novel type of adherence factors of non pathogenic microorganisms as detailed below or the presence of a DNA sequence encoding such a protein or active part thereof. Application of the invention thus in particular circumvents the laborious and costly route of screening bacteria for the capacity to adhere to living tissue. More in particular, application of the invention circumvents the use of animals and/or human volunteers for screening purposes.

A method of screening non pathogenic microorganisms for a microorganism capable of specifically binding mucosa, said method comprising detection in a manner known per se of the presence of a particular protein on or in a microorganism on or in a culture of microorganisms, said particular protein being a protein according to any of claims 1-13 falls within the scope of protection. Alternatively a method of screening non pathogenic microorganisms for a microorganism capable of specifically binding mucosa, said method comprising detection in a manner known per se of the presence of a particular gene on or in a microorganism on or in a culture of microorganisms, said particular gene encoding a protein according to any of claims 1-13 also falls within the scope of the invention.

The invention also covers a kit suitable for detection of a non pathogenic microorganism capable of specifically binding mucosa, said kit comprising a component capable of specifically binding to a protein according to any of claims 1-13 such as an antibody. In another embodiment the invention comprises a kit suitable for detection of a non pathogenic microorganism capable of specifically binding mucosa, said kit

comprising a component capable of specifically binding to a part of a nucleic acid sequence encoding a protein according to any of claims 1-13 such as a nucleic acid probe or primer.

ii) By applying the protein or polypeptide capable of specifically binding mucosa to a human or animal or by applying a microorganism capable of expressing such a protein or polypeptide or a culture of such a microorganism to a human or an animal it now becomes possible to interfere with the adhesion of pathogenic microorganisms to mucosa or mucin. In particular it becomes possible to prevent or reduce adhesion by pathogenic microorganisms to mucosa of the urogenital tract, gastro-intestinal tract, respiratory tract and/or oral/nasal cavity of humans and animals. Particularly interesting is that the invention offers a method to efficiently and specifically interfere with the adhesion of certain classes of pathogens to bacterial receptors of the mucosa and to screen for microorganisms capable of interfering with adhesion of certain classes of pathogens. Pathogens that may now be combatted comprise both Gram positive and Gram negative microorganisms in particular those that specifically bind mucosa receptors. Examples of pathogens to be combatted comprise strains of the genera *Escherichia, Campylobacter, Haemophilus, Shigella, Vibrio, Pasteurella, Yersinia, Salmonella, Mycobacterium, Listeria, Clostridium, Staphylococcus* and viruses like rotavirus, poliovirus and measles.

The invention exploits the conclusion that the infectivity of pathogens that adhere to a mucosal receptor through an adherence factor similar to that of the adhesion protein of *L. fermentum* 104R, will be reduced by probiotic bacteria harbouring an adhesion protein with a structure like that of the adhesion protein of *L. fermentum* 104R, by specific interaction with the receptor, rather than by the more general mechanism of steric hindrance.

According to the present invention, a strategy can be devised to specifically inhibit adherence of certain pathogens (those that adhere by means of an adherence factor that is stucturally related to the adhesion protein of *L. fermentum*), by administering e.g. in food or feed or as pharmaceutical composition such adhesion proteins or, microorganisms that produce such adhesion proteins. The application can be topical, oral or intravenous in any dosage form normally applied for pharmaceutical compositions and/or feed additives. The dosage form selected will depend on the type of infectious pathogen to be combatted. The dosage form may

be solid or liquid. Certain standards with regard to purity and hygiene i.e. sterility normally applicable for such compositions must be adhered to. Such circumstances are well known to a person skilled in the art.

A composition comprising a component selected from the group of components comprising

- a protein or peptide according to any of the claims 1-21
- an expression vector according to claim 24 or 25
- a recombinant microorganism according to claim 26 or 27 or a part of said microorganism, said part expressing mucosa binding promoting activity
- a non pathogenic microorganism capable of expressing a protein or peptide according to any of claims 1-21 or a part of said microorganism, said part expressing mucosa binding promoting activity as pharmaceutically active component and a pharmaceutically acceptable carrier in a pharmaceutically acceptable dosage form is covered by the invention. A composition comprising the abovementioned components in a form suitable for use as food additive is also envisaged to fall within the scope of the invention. The use of a component selected from the group of components comprising
- a protein or peptide according to any of the claims 1-21
- an expression vector according to claim 24 or 25
- a recombinant microorganism according to claim 26 or 27 or a part of said microorganism, said part expressing mucosa binding promoting activity
- a non pathogenic microorganism capable of expressing a protein or peptide according to any of claims 1-21 or a part of said microorganism, said part expressing mucosa binding promoting activity as pharmaceutically active component in a pharmaceutical composition for prophylaxis and/or treatment of disease or illness associated with a mucosa colonising pathogenic microorganism also falls within the scope of the invention.

As will be apparent from the above a method for improving food products comprising addition of a product according to any of claims 1-21 or 24-27 and/or a non pathogenic microorganism capable of expressing a protein or peptide according to any of claims 1-21 or a part of said microorganism, said part expressing mucosa binding promoting activity to the food product forms an embodiment of the invention. Preferably such a method comprises addition of a product according to any of claims 1-21 or 24-27 to the food product.

Obviously a food product comprising a product according to any of claims 1-21 or 24-27 and/or a non pathogenic microorganism capable of expressing a protein or peptide according to any of claims 1-21 or a part of said microorganism, said part expressing mucosa binding promoting activity as additive is also covered. A food product comprising a product according to any of claims 1-21 or 24-27 as additive is a particularly suitable embodiment.

A person skilled in the art will realise that the inhibiting effect may also be obtained by addition of parts of the adherence protein, e.g. peptides derived from the 29 kD adherence protein of *L. fermentum* that are found to specifically bind to mucus and mucin. The active peptides can either be synthesized chemically or made micro-biologically by a genetically engineered microorganism. Alternatively the protein can be produced by a non recombinant or recombinant microorganism and subsequently e.g. via proteolytic digestion and optionally separation of the proteolytic fragments the desired polypeptide can be obtained. From analysis of the adhesion factor of 29 kD and the adhesion factors of the pathogenic organisms Echerichia coli and Helicobacter pylori strains and cholera toxin a consensus sequence KKXXXK (Sequence id no 30) was postulated wherein X stands for any amino acid and K stands for lysine. The 29 kD protein according to the invention comprises three such sequences. They are more or less evenly distributed over the protein molecule at positions 47-52 (KKMGLK), 173-178 (KKNSTK) and 223-238 (KKLSEK) of the mature protein. The numbering corresponds to amino acids 54-59, 180-185 and 230-235 of sequence id no. 2. of the sequence listing, in which the mature protein commences with Ala at position 8. The presence of at least one of the KKXXXK sequences, preferably two of these sequences in a protein or peptide according to the invention is preferred. Most desirably three such sequences are present. In a particular embodiment the consensus sequence will be one of the natively occurring amino acid sequences present in the 29 kD protein disclosed above. Preferably sequences corresponding to those present in their native environment will be used, such sequences can however be arrived at through genetic engineering or synthetic means generally known in the art such as through DNA synthesizers, Merrifield synthesis and cloning technology as mentioned above. Preferably such sequences will also be present in a sequence such that the tertiary structure mimics that of the native protein. This can be ascertained using computer technology in a manner known per se. Such sequences are involved in binding to negatively

charged intestinal receptors.

Microorganisms that have the GRAS status, like *Aspergillus*, *Lactobacillus* and *Lactococcus* are well suited for such purposes. A person skilled in the art will realise that other microorganisms can also be used for production of adherence factors or peptides derived thereof. However it will be preferred for applications to humans to employ GRAS organisms. Lists of GRAS organisms are readily available to a person skilled in the field of foodstuffs and/or pharmaceuticals and are incorporated herein by reference. The US FDA for example maintains a list of such organisms.

The conclusion that proteins like the adhesion promoting protein of *L. fermentum* 104R or microorganisms that produce a protein with a structure similar to that of the adhesion promoting protein of *L. fermentum* 104R will interfere with specific adhesion of pathogens carrying an adhesion protein with a similar structure, does not necessarily imply that such adhesion promoting proteins or adhesion promoting protein producing microorganisms will not interfere with the adhesion of pathogens that do not produce an adherence factor with a similar structure. A person skilled in the art will immediately realise that a corrolary of the use of microorganisms with an adherence promoting protein like that of *L. fermentum* 104R might be that adherence of such bacteria to a specific receptor will also limit the adherence of pathogens with adherence factors other than the *L.fermentum*-like adhesion factor, by a general mechanism of steric hindrance. Thus the pathogenic microorganisms that can be combatted do not only comprise microorganisms that bind the mucosal receptor specifically bound by the adherence factor from the non pathogenic organism.

iii) As the group of proteins exhibiting the desired activity is now known and amino acid sequences and nucleic acid sequences have been determined it is now possible to develop and/or select microorganisms capable of improved production i.e. overexpression of the desired protein or polypeptide. This can be achieved via normal optimalisation of cultivation conditions, via selection of strains expressing proteins with improved receptor binding properties in a manner known per se.

It is also possible via genetic engineering to incorporate the nucleic acid sequence or nucleic acid sequences in microorganisms of choice that thus become capable of (over)expression and preferably also secretion of mucosa binding promoting component. Preferably the

microorganism will be a GRAS organism such as a lactic acid bacterium. It is also possible to incorporate the encoding sequences or sequences such that they are operably linked to regulating sequences that enable higher expression than with the regulating sequence normally associated with the encoding sequence. A number of high expression vectors are known for various microorganisms in particular GRAS microorganisms such as lactic acid bacteria. Recombinant microorganisms capable of expressing or overexpressing the polypeptide or protein capable of promoting the binding of mucosa of the novel group of adherence factors from non pathogenic microorganisms or recombinant expression vectors comprising the appropriate nucleic acid also fall within the scope of the invention. The microorganism that is genetically engineered may already express the adherence factor but the microorganism may also be selected from a group that does not natively express an adherence protein of the novel group. The microorganism may simply be used as production plant for the protein or polypeptide which may subsequently be isolated and applied as pharmaceutical or as food/feed additive, or the microorganism itself may be used as pharmaceutical or as food/feed additive. Preferably the protein or polypeptide producing microorganism will be non pathogenic. In particular GRAS microorganisms are preferred in order to enable applications of the expression product and/or microorganisms as active component of a pharmaceutical composition or food/feed additive.

The nucleic acid sequences may be incorporated onto a plasmid vector or integrated into the chromosome in any embodiment known per se in the recombinant DNA technology field. A large number of transformation and expression vectors and technologies are known in the state of the art and are currently also commercially available. Preferred are food-grade transformation and expression vectors and methods of transformation suitable for GRAS microorganisms.

Preferably the microorganisms to be selected and/or transformed have the following characteristics:

- Survival of the environmental conditions at the location where it must be active
- Proliferation and/or colonisation at the location where it is active
- No immune reaction against the probiotic strain
- No pathogenic, toxic, allergic, mutagenic or carcinogenic reaction by the probiotic strain itself, its fermentation products or its cell components after decease of the bacteria

- Genetically stable, no plasmid transfer
- Easy and reproducible production
- Viable during processing and storage

In general terms a recombinant microorganism is claimed comprising a nucleic acid sequence according to claim 23 and/or an expression vector according to claim 24 or 25, said nucleic acid sequence and/or expression vector being absent or in the alternative being present in a lower copy number or being expressed to a lower degree in the corresponding non recombinant microorganism. In a further embodiment the invention comprises a recombinant microorganism as just defined (according to claim 26), said microorganism being a non pathogenic microorganism, preferably indigenous to the microflora of a human or animal, more preferably to the microflora of a human.

The invention also encompasses a nucleic acid sequence encoding any of the proteins or peptides according to any of claims 1-21 and an expression vector comprising such a nucleic acid sequence, operably linked to an expression regulating sequence, said expression vector being capable of expressing the nucleic acid in a non pathogenic microorganism such as a GRAS microorganism and said expression vector preferably comprising nucleic acid derived from a GRAS microorganism. In a further embodiment the expression vector according to the invention is a vector, wherein the expression regulating sequences are not naturally associated with the gene encoding the adherence factor from which the nucleic acid sequence is derived.

iv) As the 3D structure, amino acid sequence and nucleic acid sequence of an adherence protein have now been ascertained and the similarity between other protein groups has been determined it lies within reach of a person skilled in the art to design a protein or polypeptide exhibiting improved binding characteristics and thus improved results in pharmaceutical applications or as food/feed additive. The invention thus also covers mutant polypeptides and proteins exhibiting better mucosa binding than the protein with amino acid sequences of figure 3 and better mucosa binding activity than any of polypeptides I-V as defined in the experimental part of the subject description. The invention also comprises equivalent sequences as available in nature and as mutants i.e. nucleic acid sequences encoding protein or polypeptide having at least the mucosa binding activity of the 29 kD protein and such proteins or polypeptides as well as their application in any of the methods of the

description and/or claims.

v) Having discovered a group of proteins and peptides capable of specifically binding mucosa it also becomes possible not only to target the microorganism expressing the protein or peptide to mucosa but also to use such microorganism as carrier for targeting additional compounds such as drugs, immunomodulators or antigens for eliciting an immune reponse to the mucosa. The microorganism may be selected for already having this particular characteristic or may be genetically engineered so that it subsequently produces the desired drug, immunomodulator or antigen. It also becomes possible to develop fusion proteins or peptides comprising the mucosa binding promoting amino acid sequences and additional desired amino acid sequences or molecules with the characteristic activity of choice that has to be targeted to the mucosa. A whole line of new pharmaceutical compounds specifically targeted to the mucosa can thus be developed. The invention covers such novel microorganisms and molecules and applications thereof as pharmaceutical compositions. The invention thus also covers a method for targeting a bacterium that expresses a gene of interest, for example a gene encoding an antigen of a pathogenic organism, to specific receptors of the mucosa, thereby evoking a specific immune response against the antigen and/or modulating an immune reponse. The invention covers a fusion protein or peptide comprising a protein or peptide according to any of the claims 1-21 attached to a drug, immunomodulator or antigen of choice.

Use of a component selected from the group of components comprising

- a protein or peptide according to any of the claims 1-21
- an expression vector according to claim 24 or 25
- a recombinant microorganism according to claim 26 or 27 or a part of said microorganism, said part expressing mucosa binding promoting activity
- a non pathogenic microorganism capable of expressing a protein or peptide according to any of claims 1-21 or a part of said microorganism, said part expressing mucosa binding promoting activity

as targeting component in a pharmaceutical composition for targeting an additional pharmaceutically active component to mucosa, said additional pharmaceutical component being physically linked to the targeting component falls within the scope of the invention.

The enhancement of specific adhesion of lactobacilli to receptors of

the mucosa. according to the invention, providing the opportunity to specifically target bacteria carrying compounds of interest, for example lactobacilli expressing an antigen of a pathogenic organism or a human protein, to the cells of the mucosa, thereby modulating the immune response against the antigen/human protein is a preferred embodiment of the invention.

According to the invention, the adhesion capacity of probiotic strains may be modulated by altering the properties of the adhesion protein. Such properties may involve interaction of the adhesion protein with the mucosal receptor or interaction with other (accessory) proteins.

DETAILED DESCRIPTION OF THE INVENTION

According to the invention, use is made in particular of a protein with a Mw of 29 kD of *L. fermentum* 104R, a strain isolated from the porcine gastro-intestinal tract and/or of the DNA sequence encoding this adhesion protein, which had not been described sofar. The novel protein has adhesion promoting activities. In particular the adhesion promoting activity comprises exhibiting binding to mucosa or mucin. The adhesion protein is present on the surface and is also shed off into the culture medium by *L. fermentum* 104R.

The invention more in particular exploits a special property of the adhesion promoting protein, namely that it is structurally similar to virulence proteins of several pathogenic bacteria, e.g. to adherence factors from *Campylobacter jejuni*, *Pasteurella haemolytica* and *Mycobacterium*. These features are documented in the following paragraph. According to the invention the presence of proteins with properties similar to those of the 29 kD protein can be determined using the Western blot technique, a technique well known to persons skilled in the art.

The adhesion promoting protein from *L. fermentum* 104R belongs to a class of proteins, called Class III solute transporters, of which the histidine transporter (HisJ), glutamine transporter (GlnH) and the lysine, arginine and ornithine transporter (LAO) of *Enterobacteriaceae* are the prototypes. The 3-D structure of two of these proteins, HisJ and LAO is known. The amino acid sequence of the adhesion promoting protein of *L. fermentum* 104R shows a striking similarity with on the one hand adherence proteins of pathogens, Pebl of *C. jejuni* and LapT of *P. haemolytica*, and on the other hand with members of Class III solute

transporter proteins, like LAO and HisJ. Protein modelling has shown that the predicted 3-D structure of the *L. fermentum* adhesin is also similar to that of LAO and HisJ. Amino acids in proteins in domain I of Class III solute transporters that are essential for ligand binding are conserved among all members of this class of proteins. These amino acids were also found at similar positions in the adhesion promoting proteins of *L. fermentum* 104R and in the virulence protein of *C. jejuni*. In other words, the adhesion promoting protein from *L. fermentum* 104R has a 3-D structure which is similar to that of adherence factors of pathogens like *C. jejuni* and *P. haemolytica*.

A protein belonging to the group of novel proteins as defined according to the invention is defined as a protein obtainable from a non pathogenic microorganism, said protein having mucosa binding promoting activity and a molecular weight of 20-40 kD. Preferably the weight lies between 20-30 kD. Specific embodiments are disclosed in claims 1-13. In particular a protein according to the invention comprises one or more of the following properties:

a) a molecular weight between 20 and 40 kD
b) an amino acid sequence exhibiting more than 20 % identical amino acids and more than 40% similar amino acids with the amino acid sequence of class III solute transporters and/or virulence proteins Pebl of *C jejuni*, LapT of *P. haemolytica* and *Mycobacterium tuberculosis* or *Mycobacterium leprae* 85K complex proteins A, B and C
c) promotes the specific binding to mucosal receptors also used by any of *C jejuni*, *P. haemolytica* or *Mycobacterium*
d) has a 3D structure with 2 lobes like LAO or HisJ
e) comprises one or more amino acid sequences that are 90% or more similar to the following amino acid sequences

I) AASAVNSELVHK
II) ANFVPTK
III) DTAIQSSYNK
IV) ISALFNK
V) IAGTGTNNA, preferably of the amino acid sequences II-V.

A specific embodiment is formed by the group of proteins further characterised in that the protein exhibits the consensus sequence illustrated in figures 4 and 5. The proteins claimed as such do not comprise virulence factors of pathogenic microorganisms or Class III transporters, neither does the class of recombinant proteins comprise recombinant virulence factors or recombinant Class III reporters that

could perhaps form state of the art at the filing date of the subject patent application.

Preferably a protein belonging to the group of proteins suitable for application according to the invention will exhibit binding promoting activity for mucosal receptors used by any of *C jejuni*, *P. haemolytica* or *Mycobacterium* higher or equal to that exhibited by the 29 kD protein of *L. fermentum* 104 with the amino acid sequence of figure 3 as can be determined by the mucosa binding assay illustrated in the Example.

Since the nucleotide structure of the adhesion promoting protein is known, non pathogenic microorganisms can also be screened for the presence of DNA sequences encoding proteins with a structure similar to that of the adhesion protein of *L. fermentum* 104R. The so called equivalent sequences which will encode a protein or polypeptide exhibiting at least the same mucosa binding activity. In particular such a nucleic acid sequence is a nucleic acid sequence encoding the amino acid sequence of figure 2 corresponding to that of the 29 kD protein of *L. fermentum* 104R. A nucleic acid sequence encoding the consensus amino acid sequence of the figures 4 and 5 as such also falls within the scope of the invention. In particular a nucleic acid sequence encoding a protein of 20-40 kD comprising the amino acid consensus sequence and further corresponding to the sequence of the 29 kD sequence, the only difference being in the presence of one or more mutations resulting in substitution of amino acids by other similar amino acids such that the hydropathy profiles remain similar and no serious conformation change can be expected of the resulting protein or polypeptide falls within the scope of the invention. Such sequences are known as those wherein conservative exchange of amino acids has occurred in comparison to the sequence according to sequence id. no 2. Also the invention comprises any nucleic acid sequence capable of hybridising under stringent hybridisation conditions when carrying out a blot assay in a manner known per se. Such sequences thus comprise sequences encoded by nucleic acid sequences derivable from other non pathogenic microorganisms through cross hybridisation technology using oligonucleotide probes encoding parts of the amino acid sequence according to sequence id no 2, preferably probes in which the preferred codon usage of the microorganism to be screened has been taken into account in a manner known per se. Stringent hybridisation conditions as described for example in Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory New York Maniatis, T. Fritsch, E.F. and Sambrook, J. (1982) can be suitably

applied to obtain such equivalent sequences. The cited reference also provides information regarding a number of other standard technologies mentioned elsewhere in the description and is incorporated herein by reference. In particular sequences from non pathogenic microorganisms belonging to the genera mentioned previously in the description are preferred. Also in a preferred embodiment at least one consensus sequence according to sequence id no 30 will be present. Alternatively or in addition one of the sequences of polypeptides I-V will be present. A protein or polypeptide with the amino acid sequence of the mature protein of sequence id no 2 in which amino acids have been mutated, outside the consensus sequences, having at least the mucosa binding activity of the mature protein of sequence id no 2 is also comprised within the invention. Also any sequence combining any of the above definitions is also included within the scope of the invention and forms a preferred embodiment. It is also possible that an equivalent sequence is not derivable as such from a microorganism but can be produced in an alternative manner e.g. recombinant DNA technology, PCR etc. The above embodiments are also valid for such alternative (mutant) sequences and fall within the scope of the invention. Suitably a protein or polypeptide according to the invention will be free of cell extract and other contaminating proteins. Substantial purity is preferable i.e. more than 80% pure. The purity being sufficient for application as pharmaceutical and food additive and for achieving the activity required in the applications according to the invention.

According to the present invention, bacteria may also be screened for the presence of proteins like the *L. fermentum* 104R adhesion protein, that can adhere to non-living surfaces like plastics or metal surfaces. such screening can occur as described above using oligo probes based on the amino acid sequence of the 29 kD adhesion protein. Preferably such a probe will encode a part of a consensus sequence of the figures 4 and 5. In addition a suitable probe will comprise a sequence encoding the consensus sequence of sequence id no 30. The consensus sequence or the part thereof will be at least 5 contiguous amino acids long and preferably the probe will be comprised completely of consensus sequence. Use of a combination of such probes is also possible in order to obtain a sequence encoding a protein or polypeptide exhibiting as close an identity as possible to the 29 kD protein or active part thereof required for mucosa binding activity.

FURTHER DETAILS OF EMBODIMENTS OF THE INVENTION

i) Production and purification of adhesion protein from *Lactobacillus fermentum*

In a preferred embodiment of the present invention, the adhesion promoting protein of *L. fermentum* 104R is produced by cultivating bacteria in MRS broth or LDM medium (Conway and Kjelleberg, J. Gen. Microbiol. 135:1175-1186 1989) for 14 to 24 hours. The 29 kD adhesion protein is purified from the medium to apparent homogeneity by ammonium sulphate precipitation, gel-filtration and affinity chromatography. The adhesion promoting activity is detected in the fractions by adhesion inhibition and dot blot assays, and visualized by PAGE, SDS-PAGE and western blots using horse radish peroxidase labelled mucus or mucin. The purified protein has an estimated Mw of 29 kD, under non-denaturing conditions as well as under reducing and denaturing conditions (non gradient denaturing SDS-PAGE, using a calibration curve obtained with standard proteins, and gel-filtration chromatography, relative to the standard curve) and is sensitive to pronase, and therefore, differs from the adhesion proteins described and/or implied in EP 0 210 579 and WO 90/09398, as well as those described by Conway and Kjellenberg (J. Gen. Microbiol. 135, 1175-1186), Blomberg et al (Appl. Environm. Microbiol. 59, 34-39 1993) and Aleljung et al (Current Microbiology vol 28 (1994) p. 231-236. The proteins specifically disclosed as such in the cited references do not fall within the scope of the protection of the protein or peptide claims. The compositions specifically described as such in the cited references do not fall within the scope of protection of the composition claims. In particular application of the compositions of WO 90/09398 described specifically as such for inhibition of pathogens do not fall within the scope of the protection. Where specifically is mentioned in this paragraph this implies in the examples or following the materials and methods of the cited references. The scope of generic diclosures of such references can cover some aspects of the subject invention, which however nevertheless forms a selection invention vis a vis said reference.

The adhesion promoting protein could be extracted from the cell surface of *L. fermentum* by treatment of the bacteria with 1 M LiCl and low concentrations of lysozyme. The adhesion promoting protein, which had an affinity for both small intestine mucus and gastric mucin from pigs or mice, was released into the culture supernatant fluid after 24h of

growth.

ii) <u>Screening of microorganisms for the presence of a *L. fermentum*-like adhesion protein</u>

In another preferred embodiment of the present invention, lactobacilli are screened for the presence of an adhesion promoting protein with properties similar to those of the adhesion promoting protein from *L. fermentum*, by separating proteins from the culture medium of an overnight culture by SDS-PAGE, and Western blotting using polyclonal antibodies raised in rabbits against purified adhesion protein of *L. fermentum* 104R.

iii) <u>Screening of microorganisms for the presence of a *L. fermentum*-like adhesion protein encoding gene</u>

In another preferred embodiment of the present invention, DNA is isolated from microorganisms to be screened and subjected to PCR analysis, using sets of primers that are based on the nucleotide sequences of the *L. fermentum* 104R adhesion protein encoding gene. The products formed are analysed by standard molecular biological techniques as are described in handbooks (e.g. as cited elsewhere in this description) or commercially available kits.

iv) <u>Synthesis of adhesion promoting protein in organisms other than *L. fermentum* 104R</u>

In another specific embodiment of the present invention, the gene encoding the adhesion protein from *L. fermentum* 104R or from another selected strain, isolated by the aforementioned procedure, is cloned behind a strong, preferably inducible promoter and secretion signal encoding sequence, in a GRAS production organism like *Aspergillus niger*, *Lactobacillus* etc. The culture medium is either used as such and used as food/feed additive or pharmaceutical composition, or the adhesion promoting protein is first purified (by standard techniques) and then added to food/feed preparations or pharmaceutical compositions. The nucleic acid sequence may be adjusted such that it encodes the identical amino acid sequence of the 29 kD *L. fermentum* 104R adherence protein of figure 2 but has codons adjusted to the preferred codon usage of the host in which it is incorporated. Details of preferred codon usage are available from sources known to a person skilled in the art of nucleic acid expression.

v) Production of peptides with adhesion promoting properties

In another preferred embodiment of the present invention, peptides derived from the *L. fermentum* 104R adhesion protein that show adhesion promoting properties are synthesized chemically and used as food/feed additive. Alternatively, DNA sequences, coding for such peptides are cloned behind a strong, preferably inducible promoter in a GRAS production organism like *A. niger* or *Lactobacillus* etc. In cases where the peptide encoding sequences are cloned behind a secretion signal encoding sequence and the peptides are secreted into the medium, the medium can be used as food/feed additive. In cases where the peptides are not secreted into the medium, the entire organisms, or extracts made from such organisms, can be used as food/feed additive. Alternatively the desired proteins or polypeptides may be isolated e.g. using chromotagraphy in a manner known per se for isolating protein or polypeptide e.g. in combination with antibodies specific for the protein or polypeptide to be isolated. An antibody or antibody fragment capable of binding an epitope or protein or peptide according to any of claims 1-20 falls within the scope of the invention. Such an antibody may be a polyclonal antibody (see Example) or a monoclonal antibody. An antibody specifically disclosed in any of the above cited references is excluded from the scope of protection for antibody claims as such.

vi) Targeting of an antigen or human protein to mucosa

In another embodiment of the present invention, the ability of adhesion protein to specifically adhere to mucosal tissue is exploited to target an antigen of a pathogen to the mucosa to enhance a mucosal immune response against the antigen. For this purpose, microorganisms are constructed that are capable of synthesizing the adhesion protein and the antigen of interest. Alternatively, to modulate the immune response against human proteins for the sake of suppressing auto-immune responses, microorganisms carrying a gene encoding a human protein are genetically engineered in such a way that they synthesize an adhesion protein with properties similar to those of the *L. fermentum* adhesion protein.

EXAMPLES

i) Purification and characterization of a surface protein from *Lactobacillus fermentum* that binds to small intestine mucus and gastric mucin from pig

Spent culture fluids from 14 or 24 hour cultures were collected by

centrifuging at 6000g for 20 min and dialysing at 4ºC against ultra pure water. The retenate was concentrated by ultra filtration through a 14 KDa molecular weight cut off membrane. The high molecular weight fraction was freeze dried and stored at 4ºC. Spent culture fluid was also concentrated 10 times by hollow fibre ultrafilter and ammonium sulphate was dissolved in the concentrate (40, 60 and 100% of saturation at 4ºC). The precipitates were collected by centrifugation (18000xg/30 min), dissolved in ultra pure water and dialysed against 0.01M ammonium bicarbonate. The solutions were freeze dried and kept at 4ºC.

The freeze dried preparation from 24 hours spent culture fluid concentrated by ultra filtration was dissolved in HEPES-Hanks and filtered (0.22µm) to remove insoluble particles. A 4 ml aliquot of the solution (2.1 mg of protein) was applied to a Sephadex G 200 in XK-26 column (Pharmacia-LKB, Uppsala Sweden) for gel filtration chromatography. HEPES-Hanks buffer was used to equilibrate the column and elute the sample. The fractions in each 280 nm-absorbing peak were assayed for the capacity to bind HRP-mucin and HRP-crude mucus by dot blot assay and in the inhibition of lactobacilli binding to crude mucus in microtiter plates adhesion inhibition assay. The active fractions in each 280 nm absorbing peak were pooled, dialysed and freeze dried for SDS-PAGE and western blot analysis.

Alternative purification.

Mucin was covalently coupled to Activated CH-sepharose 4B according to the instructions of the manufacturer (Pharmacia-LKB, Biotechnology). A column C10/40 (30 ml bed volume) was packed with this adsorbent and equilibrated with HEPES-Hanks. *L. fermentum* spent culture fluid, cell extracts, or active fractions from Gel filtration chromatography were loaded throw the column. Column was washed with two bed volumes of equilibrating buffer, then successively washed with different solutions (0.1 M glycine pH 3, 0.1M tris pH 8 and 0-2 M gradient of sodium chloride) at flow rates of 6 ml h^{-1}.

The adhesion promoting activity was detected in the fractions by adhesion inhibition and dot blot assays, and was visualized by PAGE, SDS-PAGE and western blots using horse radish peroxidase labelled mucus or mucin. The adhesion promoting protein could be extracted from the cell surface of *L. fermentum* by treatment of the bacteria with 1 M LiCl and low concentrations of lysozyme. The adhesion promoting protein, which had an affinity for both small intestine mucus and gastric mucin, was

released into the culture supernatant fluid after 24h of growth. The active fraction was characterized by assessing the presence of carbohydrates in (periodic-acid Schiff stain procedure, SIGMA, and DIG glycan detection kit, Boehringer Mannheim, Germany) and the heat sensitivity of the active region of the adhesion promoting protein. The adhesion promoting activity lacked carbohydrates and remained completely biologially active, when LiCl cell extracts from *L. fermentum* were heated for 5 min at 100ºC and tested by dot blot adhesion assay.

The purified protein has an estimated Mw of 29 kD, under non-denaturing conditions as well as under reducing and denaturing conditions (SDS-PAGE, using a calibration curve obtained with standard proteins, and gel-filtration chromatography, relative to the standard curve; Figure 1).

The adhesion promoting protein was further characterized by determination of the N-terminal amino acid sequence, showing the following sequence:

AXXAVNXELV(V)(K)

When the adhesion promoting protein was digested with modified porcine trypsin and the peptides formed were purified by reverse-phase HPLC, a number of peptides were found to specifically adhere to mucus and mucin, as measured by dot blot and mucin adhesion assays. The aminoacid sequence of the peptides are: I:ANFVPTK, II:DTAIQSSYNK, III:ISALFNK, IV:IIAG(T)G(T)NNA. In these sequences X most likely represents serine (S).

ii) <u>Cloning and sequencing of adherence factor encoding protein</u>

The adhesion promoting protein gene was cloned from a genomic bank of *L. fermentum* 104R. To generate a probe with which adhesion gene sequences could be identified, oligonucleotide primers were synthesized, based on the aminoacid sequence data of the sequenced peptides of the adhesion promoting protein. These oligonucleotides were used in various combinations in PCR reaction. Oligonucleotides 42 (sense; 5'-CTI.GCI.GTI.AAC/T.TCI.GAG/A.TTG/A.GT-3') and 105 (antisense; 5'-GCC.GGGA.TCC.TTT.G/A/T/CGT.G/TGG.G/TAC.G/AAA.G/ATT.G/A/TGC-3') corresponding to the N terminal peptide and peptide I, respectively, yielded a PCR product of 183 bp flanked by *Eco*RI and *Bam*HI sites, which hybridized in a Southern blot with a 3.5 kb *Sst*I-*Pst*I chromosomal *L. fermentum* fragment. The fragment was cloned in pGEM3 in *E. coli*. The position of the adhesion encoding gene was determined by restriction

enzyme analysis and the nucleotide sequence of the relevant part of the 3.5 kb fragment was determined (Figure 2). The predicted aminoacid sequence of the adhesion protein is given in Figure 3.

iii) Analysis of the aminoacid sequence of the *L. fermentum* 104R adhesion protein

Computer assisted analysis of the aminoacid sequence of the *L. fermentum* 104R adhesion protein was carried out. Figure 4 shows that the protein shows striking similarity with the virulence proteins Peb1 from *C. jejuni* and LapT from *P. haemolytica*. Figure 5 shows that the *L. fermentum* adhesion protein also shows similarity with Class III solute transporters. Figure 6 shows that the adhesion protein shows similarity to 85 K complex virulence proteins of *Mycobacterium leprae* and *Mycobacterium tuberculosis*. Protein modelling studies indicate that the predicted 3-D structure of the adhesion protein of *L. fermentum* 104R is similar to that of LAO and HisJ. These studies also indicate that Peb1 has a 3-D structure which is similar to that of LAO and HisJ.

iv) Determination of adhesion protein-like proteins in *Lactobacillus* strains

Nearly 20 *Lactobacillus* strains were cultivated in LDM medium, the culture medium was collected and the proteins separated by SDS-PAGE. The presence of adhesion protein-like protein was determined by Western blotting according to standard molecular biological techniques. The results, which are presented in Table 1, show that some *Lactobacillus* strains do produce an adhesion protein-like protein whereas others don't.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 SDS-PAGE and Western blot of the adhesion promoting protein (APP) using HRP labelled mucus for blotting. A) Molecular weight markers (lane 1); APP after affinity chromatography (lanes 2, 6 and 7); APP from native PAGE (lanes 3, 4 and 5). B) Molecular weight markers (lane 1); APP from gel-filtration chromatography (lanes 2 and 3). Arrow in lane 3 indicates position of APP; C) Western blot of APP from SDS-PAGE after gel-filtration chromatography (lane 1); after affinity chromatography (lane 2); 1M LiCl extraction of *L. fermentum* after 14 h of growth (lane 3); from PAGE (lane 4).

Figure 2 Nucleotide sequence of the adhesion promoting protein of *L. fermentum* 104R. The open reading frame starts at nucleotide 1 and ends at nucleotide 734.

Figure 3 Amino acid sequence of the adhesion promoting protein of *L. fermentum* 104R.

Figure 4 Comparison of the amino acid sequences of the adhesion promoting protein of *L. fermentum* 104R, Peb1 from *C. jejuni* and LapT from *P. haemolytica*. A consensus sequence is given below the sequences. Bold letters indicate identical aminoacids or conserved substitutions.

Figure 5 Comparison of the aminoacid sequences of the adhesion promoting protein of *L. fermentum* 104R and Class III solute transport proteins (Atunop, nopaline of *Agrobacter tumefaciens*; Atuoct, octopine *Agrobacter tumefaciens*; GlnH, glutamine binding protein of *E. coli*; HisJ, histidine binding protein, LAO, lysine, arginine, ornithine binding protein of *Salmonella thyphimurium*. A consensus sequence is given below the sequences. Aminoacids in adhesion promoting protein that also occur in other proteins are indicated in bold capital letters; colons indicate a conserved substitution and asterics a less conserved substitution.

Figure 6 Comparison of the aminoacid sequences of the adhesion promoting protein of *L. fermentum* 104R and proteins of the 85K complex of *Mycobacterium*. A consensus sequence is given below the sequences. Aminoacids that are identical in adhesin and in one or more *Mycobacterium* proteins are indicated in bold capital letters. Conserved substitutions are indicated with a colon, and less conserced substitutions with an asterisc.

Table 1 Western blot of culture medium of *Lactobacillus* strains using antibodies raised against *L. fermentum* 104R adhesion promoting protein as a probe

	Signal
L. gasseri NCK 89	+
L. reuteri ML1	++
L. murinus	+
L. fermentum 2399	+/-
L. plantarum	++
L. fermentum KLD	-
L. animalis 364T	+
L. animalis 364	+/-
L. casei ATCC 393	+/-
L. acidophilus NCK 65	-
L. animalis 362	-
L. plantarum 8014	+/-
L. plantarum LP80	+
L. brevis R3	+
L. brevis ML12	+
E. coli	-
L. fermentum 104R	++
L. plantarum 256	++

CLAIMS

1. A protein obtainable from a non pathogenic microorganism, said protein having mucosa binding promoting activity and a molecular weight of 20-40 kD, preferably 20-30 kD or an equivalent polypeptide thereof.

2. A protein or polypeptide according to claim 1, wherein the mucosa binding promoting activity comprises promoting binding to a receptor recognised on mucosa by a pathogenic microorganism, preferably any of the pathogens *Campylobacter*, *Mycobacterium* or *Pasteurella*, such as *C. jejuni*, *P. haemolytica*.

3. A protein or polypeptide according to any of the preceding claims, wherein the mucosa binding promoting activity is equal to or exceeds that of *L. fermentum* 104R 29 kD adherence factor.

4. A protein or polypeptide according to any of the preceding claims, wherein the protein or polypeptide comprises an amino acid consensus sequence as illustrated in figure 4 and/or figure 5.

5. A protein or polypeptide according to any of the preceding claims, wherein the protein or polypeptide comprises an amino acid consensus sequence as illustrated in sequence id no 30.

6. A protein or polypeptide according to any of the preceding claims, wherein the protein or polypeptide comprises one or more amino acid sequences which are more than 80%, preferably more than 90% identical to the following amino acid sequences

I) AASAVNSELVHK

II) ANFVPTK

III) DTAIQSSYNK

IV) ISALFNK

V) IAGTGTNNA, more preferably more than 80%, preferably more than 90% identical to one or more of the amino acid sequences II-V.

7. A protein or polypeptide according to any of the preceding claims, wherein the protein or polypeptide comprises one or more of the following amino acid sequences

I) AASAVNSELVHK

II) ANFVPTK

III) DTAIQSSYNK

IV) ISALFNK

V) IAGTGTNNA more preferably comprises one or more of the amino acid sequences II-V.

8. A protein or polypeptide according to any of the preceding claims, wherein the protein or polypeptide has a two lobe structure.

9. A protein or polypeptide according to any of the preceding claims, wherein the protein or polypeptide has an amino acid sequence more than 20% identical to the amino acid sequence of any of the following proteins *Atuoct*, *Atunop*, *GlnH*, *HisJ*, *LAO*.

10. A protein or polypeptide according to any of the preceding claims, wherein the protein or polypeptide comprises an amino acid sequence more than 40% identical to the amino acid sequence of any of the following proteins *Atuoct*, *Atunop*, *GlnH*, *HisJ*, *LAO*.

11. A protein or polypeptide according to any of the preceding claims wherein the protein or polypeptide comprises an amino acid sequence more than 40% identical to the amino acid sequence of *L. fermentum* 104R adherence factor of 29 kD of figure 3, preferably more than 60%, more preferably more than 80%.

12. A protein or polypeptide according to any of the preceding claims wherein the protein or polypeptide comprises an amino acid sequence identical to the amino acid sequence of *L. fermentum* 104R adherence factor of 29 kD of figure 3.

13. A protein or polypeptide according to any of the preceding claims obtainable by binding to an antibody either polyclonal or monoclonal raised against the protein or polypeptide with the amino acid sequence of *L. fermentum* 104R adherence factor of 29 kD of figure 3.

14. Polypeptide fragment being a fragment of a protein or polypeptide according to any of the preceding claims of at least 5 and preferably 7 amino acids not necessarily having mucosa binding promoting activity, the

at least 5 and preferably 7 amino acids of said polypeptide fragment being an amino acid sequence present as a contiguous sequence in any of the protein or polypeptides according to the preceding claims.

15. Polypeptide fragment according to claim 14 with a length less than 60 amino acids.

16. Polypeptide fragment according to claim 15 wherein the fragment has a length less than 40 amino acids.

17. Polypeptide fragment according to any of claims 14-16 comprising an amino acid sequence of at least a sequence of 5 amino acids present in the consensus sequence of sequence id no 30 as a contiguous sequence.

18. Polypeptide fragment according to any of claims 14-17 selected from peptides comprising an amino acid sequence at least 80% identical, preferably at least 90% to an amino acid sequence selected from peptides
I) AASAVNSELVHK
II) ANFVPTK
III) DTAIQSSYNK
IV) ISALFNK
V) IAGTGTNNA
VI) the consensus sequence of figure 4 and
VII) the consensus sequence of figure 5, preferably peptides II-VII.

19. Polypeptide fragment according to any of claims 14-18 comprising an amino acid sequence identical to an amino acid sequence selected from peptides
I) AASAVNSELVHK
II) ANFVPTK
III) DTAIQSSYNK
IV) ISALFNK
V) IAGTGTNNA
VI) the consensus sequence of figure 4 and
VII) the consensus sequence of figure 5

20. Polypeptide fragment being a combination of polypeptide fragments according to any of claims 14-19.

21. Recombinant protein, polypeptide or polypeptide fragment comprising the amino acid sequence of any of the proteins, polypeptides or polypeptide fragments according to any of the preceding claims.

22. Fusion protein or polypeptide comprising a protein, polypeptide or polypeptide fragment having mucosa binding promoting activity according to any of the preceding claims attached to a drug, an immunomodulator or antigen of choice.

23. Antibody or antibody fragment capable of binding an epitope on a protein, polypeptide or polypeptide fragment according to any of claims 1-21, preferably according to any of claims 1-13, preferably a monoclonal antibody.

24. Nucleic acid sequence encoding any of the proteins, polypeptides or polypeptide fragments according to any of claims 1-22.

25. Expression vector comprising nucleic acid according to claim 24, operably linked to an expression regulating sequence, said expression vector being capable of expressing the nucleic acid according to claim 21 in a non pathogenic microorganism such as a GRAS microorganism and said expression vector preferably comprising nucleic acid derived from a GRAS microorganism.

26. Expression vector according to claim 25, wherein the expression regulating sequences are not naturally associated with the gene encoding the adherence factor from which the nucleic acid sequence is derived.

27. Recombinant microorganism comprising a nucleic acid sequence according to claim 24 and/or an expression vector according to claim 25 or 26, said nucleic acid sequence and/or expression vector being absent or being present in a lower copy number or being expressed to a lower degree in the non recombinant microorganism.

28. Recombinant microorganism according to claim 27, said microorganism being a non pathogenic microorganism, preferably indigenous to the microflora of a human or animal, more preferably to the microflora of a human.

29. Composition comprising a component selected from the group of components comprising

- a protein or polypeptide or polypeptide fragment according to any of the claims 1-21, preferably according to any of claims 1-13, said protein or polypeptide or polypeptide fragment having mucosa binding promoting activity,
- an expression vector according to claim 25 or 26
- a recombinant microorganism according to claim 27 or 28 or a part of said microorganism, said part expressing mucosa binding promoting activity
- a non pathogenic microorganism capable of expressing a protein or polypeptide or peptide according to any of claims 1-22 or a part of said microorganism, said part expressing mucosa binding promoting activity

as pharmaceutically active component and a pharmaceutically acceptable carrier in a pharmaceutically acceptable dosage form.

30. Use of a component selected from the group of components comprising

- a protein or polypeptide or polypeptide fragment according to any of the claims 1-22 having mucosa binding promoting activity,
- an expression vector according to claim 25 or 26
- a recombinant microorganism according to claim 27 or 28 or a part of said microorganism, said part expressing mucosa binding promoting activity
- a non pathogenic microorganism capable of expressing a protein or polypeptide or peptide according to any of claims 1-22 or a part of said microorganism, said part expressing mucosa binding promoting activity

as pharmaceutically active component in a pharmaceutical composition for prophylaxis and/or treatment of disease or illness associated with a mucosa colonising pathogenic microorganism.

30. Use of a component selected from the group of components comprising

- a protein or polypeptide or polypeptide fragment according to any of the claims 1-22 having mucosa binding promoting activity,
- an expression vector according to claim 25 or 26
- a recombinant microorganism according to claim 27 or 28 or a part of said microorganism, said part expressing mucosa binding promoting activity

- a non pathogenic microorganism capable of expressing a protein or polypeptide or peptide according to any of claims 1-22 or a part of said microorganism, said part expressing mucosa binding promoting activity
as targeting component in a pharmaceutical composition for targeting an additional pharmaceutically active component to mucosa, said additional pharmaceutical component being physically linked to the targeting component.

32. A method for improving food products comprising addition of a product according to any of claims 1-22 or 25-28 and/or a non pathogenic microorganism capable of expressing a protein or polypeptide or polypeptide fragment according to any of claims 1-22 or a part of said microorganism, said part expressing mucosa binding promoting activity to the food product.

33. A method for improving food products comprising addition of a product according to any of claims 1-22 or 25-28 to the food product.

34. Food product comprising a product according to any of claims 1-22 or 25-28 and/or a non pathogenic microorganism capable of expressing a protein or polypeptide or peptide according to any of claims 1-22 or a part of said microorganism, said part expressing mucosa binding promoting activity as additive.

35. Food product comprising a product according to any of claims 1-22 or 25-28 as additive.

36. A method of screening non pathogenic microorganisms for a microorganism capable of specifically binding mucosa, said method comprising detection in a manner known per se of the presence of a particular protein or polypeptide on or in a microorganism or in a culture of microorganisms, said particular protein or polypeptide being a protein or polypeptide according to any of claims 1-22.

37. A method of screening non pathogenic microorganisms for a microorganism capable of specifically binding mucosa, said method comprising detection in a manner known per se of the presence of a particular gene in a microorganism, said particular gene encoding a protein or polypeptide according to any of claims 1-22.

38. A kit suitable for detection of a non pathogenic microorganism capable of specifically binding mucosa, said kit comprising a component capable of specifically binding to a protein or polypeptide according to any of claims 1-22 such as an antibody.

39. A kit suitable for detection of a non pathogenic microorganism capable of specifically binding mucosa, said kit comprising a component capable of specifically binding to a part of a nucleic acid sequence encoding a protein or polypeptide according to any of claims 1-22 such as a nucleic acid probe or primer.

fig-1a

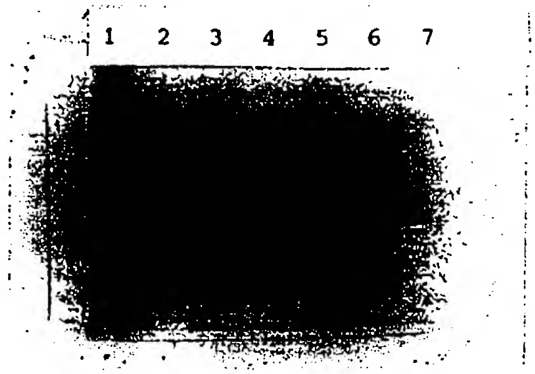

fig-1b

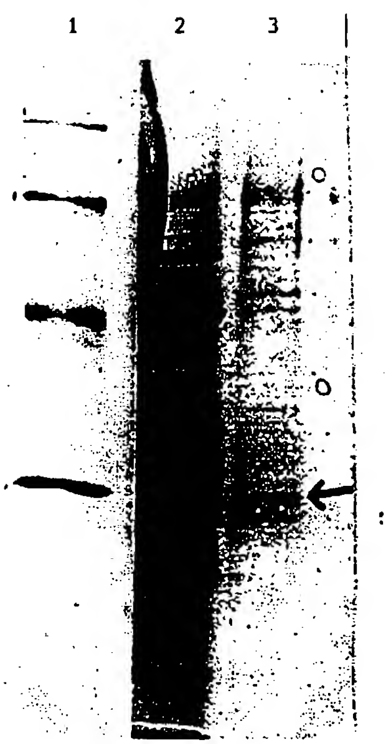

fig-1c

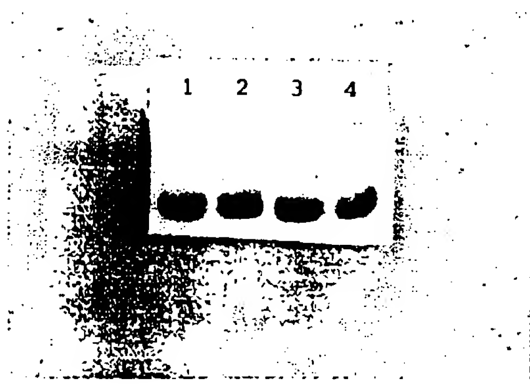

Fig-2

1 CTGCAGGAAT CACAAGTGTT TCTGCTGCTT CAGCTGTTAA TTCAGAATTA

51 GTTCATAAGG GAGAATTAAC AATTGGTCTT GAGGGAACGT ACTCTCCGTA

101 CTCTTATCGT AAAAATAACA AATTAACTGG CTTTGAAGTA GATCTTGGTA

151 AAGCAGTTGC TAAAAAGATG GGCTTAAAAG CTAACTTTGT ACCAACTAAA

201 TGGGATTCGC TAATTGCCGG TCTTGGTTCA GGTAAGTTTG ATGTAGTAAT

251 GAACAACATT ACACAGACAC CTGAACGGGC CAAGCAATAT AATTTCTCTA

301 CCCCATATAT CAAGTCCCGG TTTGCATTAA TTGTTCCTAC TGATAGTAAC

351 ATCAAAAGCT TGAAGAATAT TAAAGGCAAG AAGATTATTG CTGGTACGGG

401 AACTAATAAT GCGAATGTGG TAAAAAAATA TAAGGGTAAC CTTACACCAA

451 ATGGCGATTT TGCTAGTTCC TTAGATATGA TCAAGCAAGG TCGGGCTGCC

501 GGGACAATTA ACTCCCGTGA AGCTTGGTAC GCTTACAGCA AGAAGAACAG

551 TACTAAGGGT CTCAAGATGA TTGATGTTTC TAGTGAACAA GATCCAGCTA

601 AGATTTCAGC ACTTTTTAAC AAGAAAGATA CTGCTATTCA ATCTTCCTAC

651 AACAAGGCAC TTAAGGAACT TCAACAAGAC GGAACAGTCA AGAAGCTATC

701 TGAAAAGTAC TTCGGTGCAG ATATTACTGA ATAATTAAAA AAGATCT

Fig-3

1 AGITSVSAAS AVNSELVHKG ELTIGLEGTY SPYSYRKNNK LTGFEVDLGK

51 AVAKKMGLKA NFVPTKWDSL IAGLGSGKFD VVMNNITQTP ERAKQYNFST

101 PYIKSRFALI VPTDSNIKSL KNIKGKKIIA GTGTNNANVV KKYKGNLTPN

151 GDFASSLDMI KQGRAAGTIN SREAWYAYSK KNSTKGLKMI DVSSEQDPAK

201 ISALFNKKDT AIQSSYNKAL KELQQDGTVK KLSEKYFGAD ITE*

Fig-4.1

```
         1                                                50
Adhesin  .......... ........AG ITSVSAASAV NSELVHKGEL TIGLEGTYSP
   LapT  .......... .......... MKKTLLTLLF GCVVTAQAQD IIVMEPSYPP
   Peb1  MVFRKSLLKL AVFALGACVA FSNANAAEGK LESIKSKGQL IVGVKNDVPH
                               :  * A        :  KG:L *IG:***Y:P

         51                                              100
Adhesin  YSY.RK.NNK LTGFEVDLGK AVAKKM...G LKANFVPTKW DSLIAGLGSG
   LapT  FEMTEE.KGE IIGFDVDIAN AICKEM...N ANCTFHSQPF DSLIQSLKQK
   Peb1  YALLDQATGE IKGFEVDVAK LLAKSILGDD KKIKLVAVNA KTRGPLLDNG
         Y::  : *:* :*GFEVD::K  :AK*M      K**:V:  : DSLI*:L  G

         101                                             150
Adhesin  KFDVVMNNIT QTPERAKQYN FSTPYIKSRF ALIVPTDSNI KSLKNIKGKK
   LapT  QFDAAISGMG ITEPRKKQVL FSEPYFPSSA AFIAKKDTDF AKVKTI...G
   Peb1  SVDAVIATFT ITPERKRIYN FSEPYYQDAI GLLVLKEKKY KSLADMKGAN
         *FD:V:**:T  TPER KQYN FS*PY::S   ALIV *D*:: KSLK:IKG :

         151                                             200
Adhesin  I...IAGTGT NNANVVKKY. ..KGNLTPNG DFASSLDMIK QGRAAGTINS
   LapT  V...QNGTTY QHYLAKEKK. ..EYNVKSYA SYQNAILDVQ NGRIDAIFGD
   Peb1  IGVAQAATTK KAIGEAAKKI GIDVKFSEFP DYPSIKAALD AKRVDAFSVD
         I    AGT:  :*     K     * N:** : D:*S*:  :* *GR:*: : *
```

Fig-4.2

```
         201                                                  250
Adhesin  REAWYAYSKK NSTKGL...K MIDVSSEQDP AKISALFNKK DTAIQSSYNK
   LapT  VPVLAEMARK HEGLDFVGEK INNPNYFGDG LGIATHL..K NQVLVDQFNA
   Peb1  KSIL...... ...LGYVDDK SEILPDSFEP QSYGIVTKKD DPAFAKYVDD
             :    :K : : G:   K : : *    DP   I:*:: KK D A: * :N

         251                      275
Adhesin  ALKELQQDGT VKKLSEKYFG ADITE
   LapT  ALKTIKENGE YQKIYDKWMG GK...
   Peb1  FVKE..HKNE IDALAKKWGL .....
         ALKEI:::G* :*KL :K::G
```

Fig-5.1

```
         1                                                 50
   HisJ  ......MKKL ALSLSLVLAF SSATAAFAAI PQK.IRIGTD PTYAPFESKN
    LAO  ......MKKT VLALSLLIGL GATAASYAAL PQT.VRIGTD TTYAPFSSKD
 Atunop  MKFFNLNALA AVVTGVLLAA GPTQ...AKD YKS.ITIATE GSYAPYNFKD
 Atuoct  .....MKLKT ILCAALLLVA GQAA...AQE .KS.ITIATE GGYAPWNFSG
   GlnH  .......MKS VLKVSLAALT LAFAVSSHAA DKK.LVVATD TAFVPFEFKQ
Adhesin  .......... ...AGITSVS AASAVNSELV HKGELTIGLE GTYSPYSYRK
                                          K  LTIG E GTY:PYS *
         51                                                100
   HisJ  AQGELVGFDI DLAKELCKRI NTQCTFVENP LDALIPSLKA KKIDAIMSSL
    LAO  AKGEFIGFDI DLGNEMCKRM QVKCTWVASD FDALIPSLKA KKIDAIISSL
 Atunop  AGGKLIGFDI DLGNDLCKRM NIECKFVEQA WVGIIPSLTA GRYDAIMAAM
 Atuoct  PGGKLDGFEI DLANALCEKM KAKCQIVAQN WDGIMPSLTG KKYDAIMAAM
   GlnH  .GDKYVGFDV DLWAAIAKEL KLDYELKPMD FSGIIPALQT KNVDLALAGI
Adhesin  .NNKLTGFEV DLGKAVAKKM GLKANFVPTK WDSLIAGLGS GKFDVVMNNI
            KL GFEV DLGKA:AKKM  LK**FVP   WD:LI::L*: GK:D::M  I
         101                                               150
   HisJ  SITEKRQQEI AFTDKLYA.. ......ADSR LV........ .......VAK
    LAO  SITDKRQQEI AFSDKLYA.. ......ADSR LI........ .......AAK
 Atunop  GIQPAREKVI AFSRPYLLTP MTFLTTADSP LLKTQVAIEN LPLDNIAPEQ
 Atuoct  SVTPKRQEVI GFSIPYAAGI NGFAVMGDSK LAEMPGLGET YSLDSQADAA
   GlnH  TITDERKKAI DFSDGYYKSG LLVMVKANNN DV........ ..........
Adhesin  TQTPERAKQY NFSTPYIKSR FALIVPTDSN I......... ..........
         T TPER K:   :FS PY KS   : ::V  DSN :
         151                                               200
   HisJ  NSDIQPTVAS LKGKRVGVLQ GTTQETFGNE HWAPKGIEIV SYQGQDNIYS
    LAO  GSPVQPTLES LKGKHVGVLQ GSTQEAYAND NWRTKGVDVV AYANQDLIYS
 Atunop  KAELDKFTKI FEGVKFGVQA GTSHEAFM.K QMMP.SVQIS TYDTIDNVVM
 Atuoct  KKAIADISSF LNGTTVGVQG STTASTFLDK YFKG.SVDIK EYKSVEEHNL
   GlnH  .....KSVKD LDGKVVAVKS GTGSVDYAKA NIKTK..DLR QFPNIDNAYM
Adhesin  .....KSLKN IKGKKI.IAG TGTNNANVVK KYKGNLTPNG DFASS....L
              KSLK  :KGKK: : G ::T  A   K  :KG        FAS     L
```

Fig-5.2

```
         201                                                    251
HisJ     DLTA.GRIDAA FQDEVAASEG FLKQPVGKDY KFGGPAVKDE KLFGVGTGMG
LAO      DLTA.GRLDAA LQDEVAASEG FLKQPAGKEY AFAGPSVKDK KYFGDGTGVG
Atunop   DLKA.GRIDAS L.ASVSFLKP LTDKPDNKDL KMFGPRMTGG .LFGKGVGVG
Atuoct   DLTS.GRLDAV L.ANATVLAA AIEKPEMKGA KLVGPLFSGG .EFG.VVAVG
GlnH     ELGTN.RADAV LHDTPNILY. FIKTAGNGQF KAVGDSLEAQ QY.....GIA
Adhesin  DMIKQGRA.AG TINSREAWYA YSKKNSTKGL KMIDVSSEQD ...PAKISAL
         D:   GRA A:    :S A:YA : KK   KGL KM:: S E::    *  ::
         252                                     291
HisJ     LRKEDNELRE ALNKAFAEMR ADGTYEKLAK KYFDFDVYGG.
LAO      LRKDDTELKA AFDKALTELR QDGTYDKMAK KYFDFNVYGD.
Atunop   IRKEDADLKA LFDKAIDAAI ADGTVQKLSQ QWFGYDASPKQ
Atuoct   LRKEDTALKA DFDAAIKAAS EDGTIKTLSL KWFKVDVTPQ.
GlnH     FPKGSDELRD KVNGALKTLR ENGTYNEIYK KWFGTEPK...
Adhesin  FNKKDTAIQS SYNKALKELQ QDGTVKKLSE KYFGADITE.
         F K*DTA::* *:NKALKEL: QDGTVKKLS  KYFG D:T
```

Fig-6.1

```
          1                                                 50
Mtu85c    MTFFEQVRRL RSAATTLPRR VAIAAMGAVL VYGLVGTFGG PATAGAFSRP
Mlep85c   MKFLQQMRKL FGLAAKFPAR LTIAVIGTAL LAGLVGVVGD TAIAVAFSKP
Mtu85b    ..MTDVSRKI RA....WGRR LMIGTAAAVV LPGLVGLAGG AATAGAFSRP
Mlep85b   ..MIDVSGKI RA....WGRW LLVGAAAT.. LPSLISLAGG AATASAFSRP
Mlep85a   .......... .......... .......... .......... ..........
Adhesin   .......... .......... .......... .AGSTSVSAA SAVNSELVHK
                                            :G *::*:: *A:*:*::**

          51                                               100
Mtu85c    G.LPVEYLQV PSA.SMGRDI KV.QFQGGGP ..HAVYLLDG LRAQDDY..N
Mlep85c   G.LPVEYLQV PSP.SMGHDI KI.QFQGGGQ ..HAVYLLDG LRAQEDY..N
Mtu85b    G.LPVEYLQV PSP.SMGRDI KV.QFQSGGN NSPAVYLLDG LRAQDDY..N
Mlep85b   G.LPVEYLQV PSE.AMGRTI KV.QFQNGGN GSPAVYLLDG LRAQDDY..N
Mlep85a   .......... .......... .......... .......... ..........
Adhesin   GELTIG.LET YSPYSYRKNN KLTGFEVDGK ...AVAKKMG LKA..NFVPT
          G L*:: L:*  Sp S  ::  K: *F: :G*    AV    G L:A  ::  *

          101                                              150
Mtu85c    GWDINTPAFE EYYQSG.LSV IMPVGGQSSF YTDWYQPSQS NGQNYTYKWE
Mlep85c   GWDINTPAFE EYYHSG.LSV IMPVGGQSSF YSNWYQPSQG NGQHYTYKWE
Mtu85b    GWDINTPAFE WYYQSG.LSI VMPVGGQSSF YSDWYSPACG KAGCQTYKWE
Mlep85b   GWDINTSAFE WYYQSG.LSV VMPVGGQSSF YSDWYSPACG KAGCTTYKWE
Mlep85a   .......... .......... .......... .......... ..........
Adhesin   KWSL.IAGLG ....SGKFDV VM........ .NNITTPERA KQ....YNFS
           W*: *::::     SG :*V VM          *N  *P**: K*    Y*:*

          151                                              200
Mtu85c    T.FLTREMPA WLQANKGVSP TGNAAVGL.. ....SMSGGS ...ALILAAY
Mlep85c   T.FLTQEMPS WLQANKNVLP TGNAAVGL.. ....SMSGSS ...ALILASY
Mtu85b    T.FLTSELPQ WLSANRAVKP TGSAAIGL.. ....SMAGSS ...AMILAAY
Mlep85b   T.FLTSELPK WLSANRSVKS TGSAVVGL.. ....SMAGSS ...ALILAAY
Mlep85a   ...LTSELPQ YLQSNKQIKP TGSAAVGL.. ....SMAGLS ...ALTLAIY
Adhesin   TPYIKS...R FL.....IVP TDSNIKSLKN IKGKKIAGTG TNNANVVKKY
          T ::*S   * :L     ::P T:S*: :L        *:AG*:    A ::  Y
```

Fig-6.2


```
          201                                              250
Mtu85c    YPQQFP...Y AASLSGFLNP SEGWWPTLIG LAMNDSGGYN ANSMWGPSSD
Mlep85c   YPQQFP...Y AASLSGFLNP SEGWWPTMIG LAMNDSGGYN ANSMWGPSTD
Mtu85b    HPQQFI...Y AGSLSALLDP SQGMGPSLIG LAMGDAGGYK AADMWGPSSD
Mlep85b   HPDQFI...Y AGSLSALMDS SQGIEPQLIG LAMGDAGGYK AADMWGPPND
Mlep85a   HPDQFI...Y VGSMSGLLDP SNAMGPSLIG LAMGDAGGYK AADMWGPSTD
Adhesin   K GNLPNGDF ASSL.DMIK. .QGR...... .....AAGIN SREAWY....
          * :*:P   : A:SL ::::   QG             A:G*N :*: W

          251                                              300
Mtu85c    PAWKRNDPMV QIPRLVANNT RIWVYCGNGT PSDLGGDNIP AKFLEGLTLR
Mlep85c   PAWKRNDPMV QIPRLVANNT RIWVYCGNGA PNELGGDNIP AKFLESLTLS
Mtu85b    PAWERNDPTQ QIPKLVANNT RLWVYCGNGT PNELGGANIP AEFLENFVRS
Mlep85b   PAWQRNDPIL QAGKLVANNT HLWVYCGNGT PSELGGTNVP AEFLENFVHG
Mlep85a   PAWKRNDFTV NVGTLIANNT RIWMYCGNGK PTELGGNNLP AKLLEGLVRT
Adhesin   .AYSKK.... .......NST K.....GLGI ..DVSSEQDP AK.ISAL.FN
           A:*:*            N*T :     G G*   D::::* P AK :*:L ::

          301                                              350
Mtu85c    TNQTFRDTYA ADGGRNGVFN FPPNGTHSWP ..YWNEQLVA MKADIQHVLN
Mlep85c   TNEIFQNTYA ASGGRNGVFN FPPNGTHSWP ..YWNQQLVA MKPDIQQILN
Mtu85b    SNLKFQDAYN AAGGHNAVFN FPPNGTHSWE ..YWGAQLNA MKGDLQSSL.
Mlep85b   SNLKFQDAYN GAGGHNAVFN LNADGTHSWE ..YWGAQLNA MKPDLQNTL.
Mlep85a   SNIKFQDGYN AGGGHNAVFN FPDSGTHSWE ..YWGEQLND MKPDLQQYL.
Adhesin   KDTAIQSSYN .....KALKE LQQDGVKKLS EKYFGADITE ..........
          *:* :Q:*YN      *A: : L*:DG***:*   Y:GA::**
          351           364
Mtu85c    GATPPAAPAA PAA*
Mlep85c   GSNNNA*... ....
Mtu85b    GAG*...... ....
Mlep85b   MAVPRSG*.. ....
Mlep85a   GATPGA*... ....
Adhesin   .......... ....
```

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 96/00409

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/31 C07K14/335 C12N15/62 C07K16/12 A61K39/07
G01N33/50 A23L1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K G01N A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CURRENT MICROBIOLOGY, 28 (4). 1994. 231-236., XP000573194 ALELJUNG P ET AL: "Purification of collagen-binding proteins of Lactobacillus reuteri NCIB 11951" see the whole document ---	1-3,20, 22, 28-30, 35,37
X	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, 61 (7). 1995. 2467-2471., XP000573172 TOBA T ET AL: "A collagen-binding S-layer protein in Lactobacillus crispatus" see the whole document ---	1-3,20, 22, 28-30, 35,37

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
24 January 1997	03.02.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Espen, J

Form PCT/ISA/210 (second sheet) (July 1992)

Inter nal Application No
PCT/NL 96/00409

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MICROBIOLGY, vol. 141, no. 7, July 1995, pages 1781-1784, XP000573094 RODRIGUEZ F ET AL.: "An operon encoding a novel ABC-type transport system in Bacillus subtilis" see the whole document ---	1-3,9, 23,24, 26-30, 35-38
X	WO,A,90 09398 (BIOINVENT INT AB) 23 August 1990 see the whole document ---	1,2
X	APPL ENVIRON MICROBIOL, DEC 1994, 60 (12) P4487-94, UNITED STATES, XP000614693 GREENE JD ET AL: "Factors involved in adherence of lactobacilli to human Caco-2 cells." see the whole document ---	1-3
X	J BIOL CHEM, FEB 11 1994, 269 (6) P4135-43, UNITED STATES, XP002023710 OH BH ET AL: "The bacterial periplasmic histidine-binding protein. structure/function analysis of the ligand-binding site and comparison with related proteins." see page 4136, left-hand column ---	1-3, 8-10, 14-17,20
X	J BIOL CHEM, JUL 7 1995, 270 (27) P16097-106, UNITED STATES, XP002023711 WOLF A ET AL: "Structure/function analysis of the periplasmic histidine-binding protein. Mutations decreasing ligand binding alter the properties of the conformational change and of the closed form." see page 16097 - page 16098 ---	1-3, 8-10, 14-17,20
A	INT J FOOD MICROBIOL, DEC 1994, 24 (1-2) P179-89, NETHERLANDS, XP000573171 HARTY DW ET AL: "Pathogenic potential of lactobacilli." see the whole document ---	1
A	J GEN MICROBIOL, 138 (12). 1992. 2657-2661., XP000614690 HENRIKSSON A ET AL: "ADHESION TO PORCINE SQUAMOUS EPITHELIUM OF SACCHARIDE AND PROTEIN MOIETIES OF LACTOBACILLUS-FERMENTUM STRAIN 104-S" see the whole document -----	

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte[illegible]nal Application No
PCT/NL 96/00409

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9009398	23-08-90	AU-A- 5174390	05-09-90
		EP-A- 0478549	08-04-92

Form PCT/ISA/210 (patent family annex) (July 1992)

WEST